Storage of *Cohniella cepula* (Orchidaceae) Pollinia: Fertilizing Ability and Subsequent Fruit and Seed Formation

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Abstract. Pollen storage is of great importance for plant breeding and production besides an efficient means for preservation of haploid gene pool of plant genetic resources and rare or endangered species. Pollinia of *Cohniella cepula* were stored over 1 year at 4, −20, −70, and −196 °C. Fertilizing ability of fresh and stored (30 to 360 days) pollinia was determined by the fruit and seed formation for each treatment, as well as by the seed viability, in vitro seed germination, and seedling growth. Pollinia stored at −70 and −196 °C showed high fertilizing ability (94.4% to 100.0%) even 1 year after collection, revealing no significant differences with fresh pollinia. Seeds from all treatments showed high viability (91.2% to 94.3%) through the 2,3,5-triphenyltetrazolium chloride (TTC) reduction assay and high in vitro germination (91.7% to 97.3%). Thus, successful ultracold storage of *C. cepula* pollinia was feasible without any desiccation, cryoprotection, or precooling treatment before placing into an ultra freezer (−70 °C) or immersing in liquid nitrogen (LN) (−196 °C).

Pollen storage is of great importance for plant breeding and production as a means of overcoming asynchrony in flowering, location, and availability of plants for crosses. Likewise, it is an efficient means for preservation of the haploid gene pool of plant genetic resources and rare or endangered species (Ganeshan et al., 2008; Hanna and Towill, 1995). Particularly for orchid breeding and propagation, the proper storage of pollen may allow crosses between plants that show temporal and spatial separation between periods of sexual reproduction (Pritchard and Prendergast, 1989; Vendrame et al., 2008). For these reasons, it is desirable to develop methods that ensure the long-term preservation of pollen. Cryopreservation, i.e., storage of samples at ultralow temperature of LN (–196 °C), is considered the best procedure for germplasm long-term preservation without genetic alterations (Ashmore, 1997). Cryopreservation of pollen has been applied to several species using different cryoprotectants (Ganeshan et al.,

2008; Grout and Roberts, 1995). However, the literature on orchid pollen cryopreservation is scarce (Popova et al., 2016). The available information indicates that air-drying and vitrification methods are both suitable treatments to enable the cryostorage of orchid pollen (Ajeeshkumar and Decruse, 2013; Pritchard and Prendergast, 1989; Vendrame et al., 2008), and most studies have been concerned with pollen storage in LN followed by rewarming and in vitro germination assessment.

The genus *Cohniella* Pfitzer (Orchidaceae, Oncidiinae) includes 13 species that are known in horticulture as the "rat-tail oncidiums." It is a Neotropical genus distributed widely from northern Mexico into southern Brazil and northern Argentina, mostly in the lowlands (Carnevali Fernández-Concha et al., 2010). *Cohniella cepula* is a wild species of Argentina, Bolivia, Brazil, Paraguay, and Peru with great ornamental potential, which is threatened because of the extensive disturbance of their natural habitat and indiscriminate harvesting of naturally growing plants. Consequently, it is imperative to develop effective propagation and preservation strategies for this orchid species. So far, there is no report about germplasm preservation or micropropagation for the genus *Cohniella*. In this study, we examined the possibility of storing *C. cepula* pollinia at 4, –20, –70, and –196 °C. Pollen viability was evaluated by their fertilizing ability,

fruit and seed formation, seed viability, in vitro seed germination, and seedling growth.

Materials and Methods

Plant material. Cohniella cepula (Hoffmanns.) Carnevali and G. Romero collection was maintained in the orchid's greenhouse from the Facultad de Ciencias Agrarias, Universidad Nacional del Nordeste (Fig. 1A). The plants used in this study came from the acclimatization of in vitro plants obtained by asymbiotic germination of seeds from different fruits acquired in local nursery gardens. The plants (2–3 years old) were nourished every 2 weeks with 0.15% Peters® Professional Blossom Booster (10–30–20 + 2MgO). Pollinaria were collected in Jan.–Feb. 2013, at the beginning of the main flowering season of this species, and immediately distributed among different treatments as described subsequently. The main flowering took place from early-Jan. to early-Apr. 2013 and bloom lasted until mid-May (summer to early autumn). During this period, we carried out pollination tests with pollen stored for 30 to 120 d. Some others plants bloomed in Sept.–Oct. 2013 (at the beginning of spring), and during this period, we tested pollination with pollen stored for 240 d. At the beginning of the next main flowering season (Jan.–Feb. 2014), we tested the fertilizing ability of pollen stored for 360 d. In addition, samples of pollinia were oven-dried at 103 °C for 17 h to constant weight, and the average moisture content (MC) of fresh pollen was determined following standard procedures described by the International Seed Testing Association (ISTA, 1993).

Storage procedure and treatments. Pollinaria from 1-d opened flowers of *C. cepula* were transferred to 2-mL Nalgene® cryovials (two pollinaria per cryovial) and distributed among four treatments: a) storage at 4 °C (refrigerator), b) storage at –20 °C (conventional freezer), c) storage at –70 °C (ultra freezer), and d) storage at –196 °C (by direct immersion in LN). Furthermore, a control treatment was established consisting of fresh pollinarium collected from one flower and immediately used to pollinate another flower from a different inflorescence of *C. cepula* (since previous studies indicated that it is an outcrossing species).

Assessment of pollen fertilizing ability. Fertilizing ability of fresh and stored pollinia was tested through hand pollinations of open flowers of *C. cepula*. After different periods of storage (30–360 d), the samples stored at frozen temperatures were rapidly rewarmed (2 min) in a 35 °C water bath. Pollinaria from each treatment were removed from cryovials (Fig. 1B) and used to pollinate open flowers from different plants. Six flowers previously emasculated were hand pollinated for each treatment. Pollinated plants were maintained in a greenhouse, and pollen fertilizing
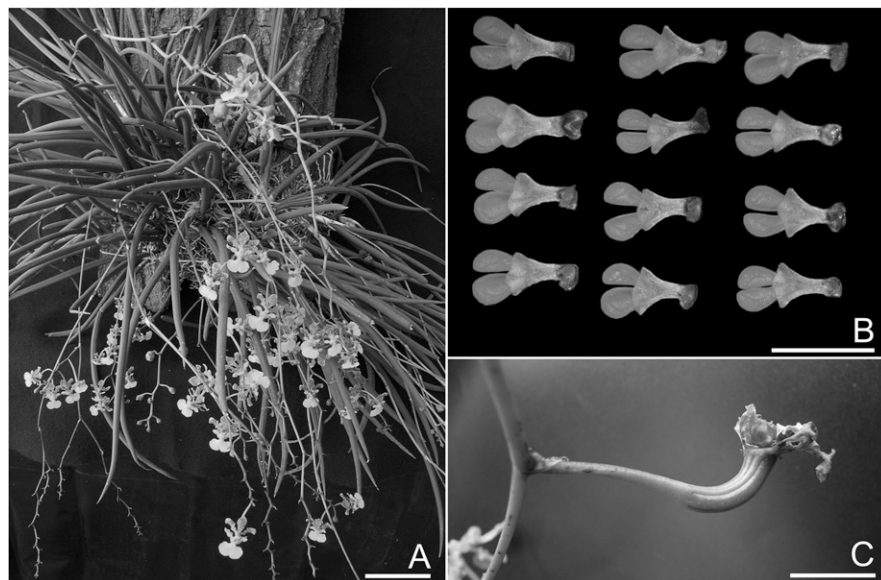
Fig. 1. (**A**) Plant of *Cohniella cepula* maintained in a greenhouse, bar = 10 cm. (**B**) Fresh (right) and 1-year-stored pollinaria at –70 (middle) and –196 °C (left), bar = 3 mm. (**C**) Thirty-day postpollination capsules obtained from cryopreserved pollinia, bar = 1 cm.

ability was determined by the fruit (capsules) and seed formation, 90 d after hand pollinations.

Seed viability, in vitro germination, and plant regeneration. Green capsules containing mature seeds were collected 90 d after hand pollination. Capsules were surface sterilized by soaking them in 70% ethanol (2 min) followed by immersion in an aqueous solution of 2.5% sodium hypochlorite (30 min) and then rinsed three times in sterile deionized water. Capsules were then opened in a laminar flow hood and seed samples were removed. Seed viability from capsules of each treatment was determined using the TTC reduction assay (Singh, 1999). Moreover, seed germination was determined by sowing seeds in 100-mL glass flasks containing 25 mL of solidified (0.65% agar A-1296; Sigma Chem. Co., St. Louis, MO) Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Medium pH was adjusted to 5.6 with KOH or HCl before the addition of agar. Flasks with culture medium were autoclaved at 1.46 kg·cm^{-2} (20 min). For sowing, seeds were suspended in previously sterilized distilled water added with 0.2% agar and 300 μL of the inoculum was dropped per glass flask (each drop containing 484 ± 22 seed), using a sterile 100–1000 μL graduated micropipette. Cultures were sealed with Resinite AF 50® (Casco S.A.I.C. Company, Buenos Aires, Argentina) and incubated in a growth room at 27 ± 2 °C with a 14-h light/10-h dark photoperiod with 116 μmol·m^{-2}·s^{-1} photosynthetic photon flux density provided by cool-white fluorescent lamps. Seed germination was monitored every 30 d for all treatments, and germination percentages were determined after 90 d based on the number of seeds which developed into protocorms. Protocorms were then transferred to 350-mL glass flasks

containing 70 mL of solidified MS medium supplemented with 0.5 g·L^{-1} activated charcoal, and were monitored for growth and shoot and root development.

Experimental design and data analysis. Storage treatments were arranged in a completely randomized design with three replicates of six pollinaria per treatment. Pollen fertilizing ability was evaluated by the percentage of flowers that were successfully pollinated showing fruit and seed formation. Seeds from three randomly selected capsules of each treatment were also analyzed for viability and germination. The data were subjected to analysis of variance and the significance of mean differences was determined using Tukey's multiple comparison test ($P < 0.05$).

Results

Pollinia from *C. cepula* showed a MC of 12.2% (fresh weight basis) after collecting. Figure 2 shows the fertilizing ability of pollinia stored up to 360 d at 4, –20, –70, and –196 °C. Fresh pollinia collected and immediately used for direct pollination showed high fertilizing ability (94.4%). For pollinia stored at 4 °C, fertilizing ability remained high (77.8%) 60 d after collection, but it markedly decreased to 40% after storage for 120 d, and fell to 0% on 240 d after collection. For pollinia stored at –20 °C, fertilizing ability remained high (88.9%) 120 d after collection, but gradually fell to 55.6% on 360 d after collection. Pollinia stored at –70 and –196 °C showed no significant differences with fresh pollinia fertilizing ability (94.4% to 100%), even 1 year after collection.

As a consequence of the high fertilizing ability of fresh and stored pollinia, successful capsule formation and seed production (Figs. 1C and 3A) were achieved. Seeds from the control treatment (flowers pollinated with

fresh pollinia) showed 92.1 ± 1.2% viability through the TTC assay and 93.3 ± 0.9% asymbiotic germination. Data in Table 1 present the results of viability and seed germination percentages from capsules of each storage treatment. Seeds showed high viability (91.2% to 94.3%) and high germination percentages (91.7% to 97.3%). No significant differences were found when seed viability and germination from all treatments (flowers pollinated with fresh and stored pollinia) were compared. Germinating seeds showed enlargement, change to green color, and development into protocorms (Fig. 3C). Protocorms developed leaf primordia and rhizoids, and successfully developed into seedlings, which then showed well-formed leaves and roots (Fig. 3D).

Discussion

Partially dehydrated pollen (PDP) with less than 30% initial MC and partially hydrated pollen (PHP) with more than 30% MC exist in flowering plants (Nepi et al., 2001). Although orchid pollen MC is least studied, the available literature (Pacini, 2009; Pacini and Hesse, 2002) suggests that all species with pollinia have PHP (MCs above 30% at anthesis). Consequently, the term "recalcitrant" has been applied as an analogy with storage behavior of seeds that are also shed at high MC and sensitive to dehydration (Franchi et al., 2002, 2011). However, pollinia of *C. cepula* have PDP (≈12% MC) at anthesis, suggesting that this orchid pollen would be not recalcitrant. In this work, pollinia tolerated the storage at temperatures of 4 and –20 °C, but their longevity was reduced at these temperatures (60 to 120 d). This indicates that deleterious physical and chemical changes proceed gradually in refrigerator-stored pollen and they are not fully detained by the colder temperature attained in a conventional freezer. Studies have indicated that pollen deterioration during aging involves disrupted intracellular integrity, decreased activity of enzymes (e.g., cytochrome oxidase), accumulation of free radicals, and de-esterification and peroxidation of membrane lipids leading to increased leakage of cellular components upon rehydration (imbibitional leakage) (Georgieva and Kruleva, 1994; Priestley et al., 1985; van Bilsen and Hoekstra, 1993). Low temperature (4 to 6 °C) storage of air-dried orchid pollen has been described in species of *Dendrobium*, *Vanda*, *Cymbidium*, and *Arachnis* by confirming pollen viability after 280 d (Shijun, 1984). However, in that study, there was no indication as to the MC levels attained by pollinia. Otherwise, air-dried pollen (≈15% MC) of *Anacamptis pyramidalis* and *Dactylorhiza fuchsii* recorded no decrease in germination after 12 months of storage at –20 °C (Pritchard and Prendergast, 1989). More recently, Marks et al. (2014) reported that pollinia of *D. fuchsii* equilibrated at 33% relative humidity (≈8% MC) and stored for 6 years at –20 °C showed a lower level of
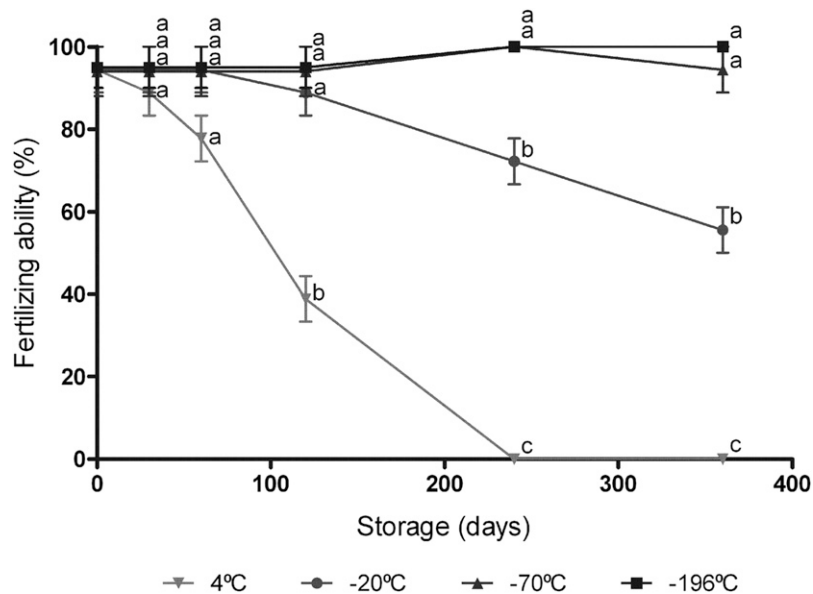
Fig. 2. Fertilizing ability of *Cohniella cepula* pollinia stored up to 360 d at 4, –20, –70, and –196 °C. Bars indicate SE. Different letters denote significant differences according to Tukey's multiple comparison test (*P* < 0.05).
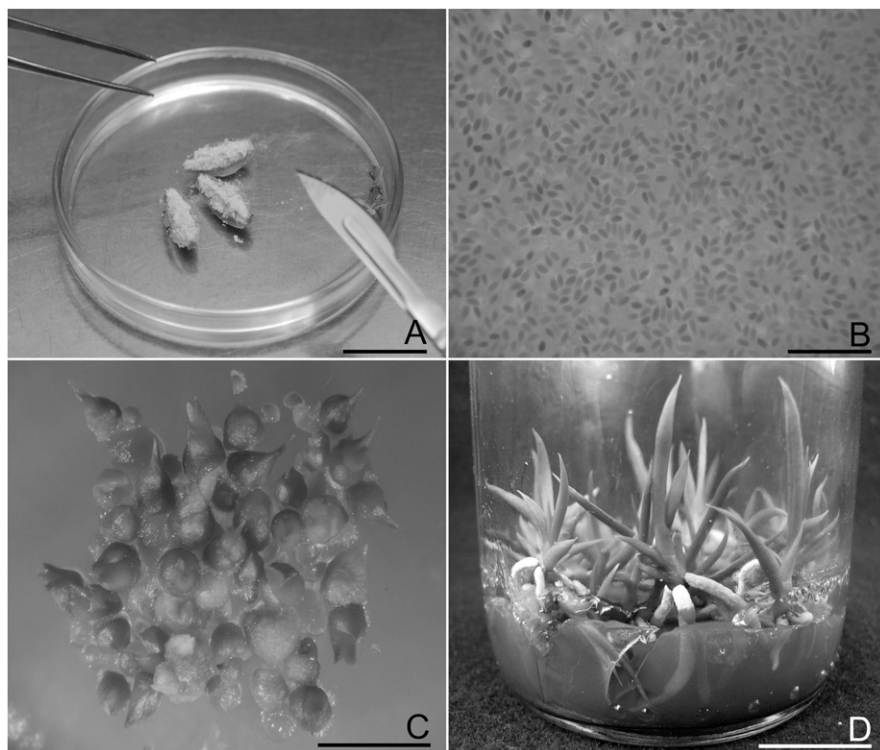

Fig. 3. (**A**) Ninety-day postpollination capsule of *Cohniella cepula* aseptically opened for removing seed samples, bar = 2 cm. (**B**) 2,3,5-Triphenyltetrazolium chloride (TTC)–positive red coloration of viable seeds observed under a stereomicroscope (4×), bar = 1 mm. (**C**) In vitro seed germination and protocorm development (90 d after seed sowing), bar = 5 mm. (**D**) In vitro seedling growth (≈150 d after seed sowing), bar = 2 cm.

germination (64%) than fresh pollen (80%), which was also expressed on a reduced seed siring ability.

Ultralow temperatures (from –70 °C to –196 °C) can be used for preserving pollen in an unaltered condition with great potential, especially in cases where long-term preservation is desired (Ajeeshkumar and Decruse, 2013). Cryopreservation is considered the best procedure, since it provides the possibility of significantly extended storage periods with the maximum genetic stability. At this temperature, all cellular divisions and metabolic processes are stopped.

The plant material can thus be stored without alteration or modification for a theoretically unlimited period of time (Ashmore, 1997; Engelmann, 2011). However, cryopreservation presents a series of problems mainly associated to the initial MC of sample and the alterations to which the material is subjected during the process of cooling/rewarming. Both factors should be evaluated for each biological material before using any cryopreservation method. The MC of tissues is the most critical factor for successful cryopreservation (Vertucci and Roos, 1993). Optimal survival is generally obtained when samples are frozen with a MC comprised between 10% and 20% (fresh weight basis) (Engelmann, 2011). Likewise, the use of proper cryoprotectants [glycerol, ethylene glycol, dimethylsulfoxide (DMSO), and plant vitrification solutions (PVS)] can increase the success of cryopreservation protocols by suppressing ice crystallization during cooling to and warming from LN (Sakai et al., 1990; Vendrame et al., 2008).

Ultracold storage of orchid pollen was first reported by Ito (1965), indicating that variously desiccated pollinia of *Dendrobium nobile*, *Dendrobium* cv. Lady Hamilton, and *Calanthe furcata* were able to germinate after 712, 975, and 718 d at –79 °C, respectively. In addition, the lifespan of pollen was enhanced substantially at this temperature compared with storage at 4 °C (at which pollen viability was lost in 6 months). Likewise, in this work, a cryoprotective mixture consisting of glycerol and ethylene glycol was successfully tested for *D. nobile* pollinia stored at –79 °C for 93 d. Pritchard and Prendergast (1989) reported no decreased germination of air-dried pollen (≈15% MC) of *A. pyramidalis* and *D. fuchsii* after 12 months of storage at –196 °C. In addition, they demonstrated the advantage of using 0.5 M DMSO as a cryoprotectant before 1-h cryostorage by programmed cooling of *Dactylorhiza maculata* and *Listera ovata* pollinia, but they indicated that such cryoprotectant was not so effective for *D. fuschii*, *A. pyramidalis*, *Gymnadenia conopsea*, and *Orchis mascula*. Moreover, germination of *G. conopsea* was markedly reduced in response to DMSO treatment without LN exposure. The use of other cryoprotectants, such as glycerol and ethylene glycol, were also less effective in those species. The PVS2 (a combination of DMSO, glycerol, sucrose, and ethylene glycol developed for the cryopreservation of plant tissues) has been suggested to cryopreserve pollen of *Dendrobium ovatum*, *Luisia macrantha*, and *Rhyncostylis retusa*, though experimental details are not provided (Ganeshan et al., 2008). Ajeeshkumar and Decruse (2013) confirmed the earlier suggestion of PVS2 exposure for the successful cryostorage of *L. macrantha* pollinia during 668 d, retaining pollen germinability equal to its initial level and allowing successful crosses with *Vanda tessellata*, generating viable seeds which germinated and

Table 1. Viability [2,3,5-triphenyltetrazolium chloride (TTC)] and in vitro germination (Germ.) percentages of *Cohniella cepula* seeds.[z] Seeds were derived of capsules from successfully pollinated flowers with pollinia stored up to 360 d at 4, –20, –70, and –196 °C.

Storage time (days)	4 °C		–20 °C		–70 °C		–196 °C	
	TTC (%)	Germ. (%)	TTC (%)	Germ. (%)	TTC (%)	Germ. (%)	TTC (%)	Germ. (%)
30	92.7 ± 2.1	93.2 ± 2.3	93.5 ± 2.2	92.7 ± 2.9	91.8 ± 1.9	93.0 ± 2.1	94.1 ± 1.5	95.9 ± 1.5
60	91.2 ± 1.9	91.7 ± 2.7	92.3 ± 2.2	94.1 ± 1.5	94.3 ± 1.6	94.1 ± 1.6	93.8 ± 2.0	94.7 ± 0.8
120	93.8 ± 1.3	94.0 ± 2.1	91.7 ± 2.5	92.7 ± 2.0	93.6 ± 2.1	94.7 ± 1.5	94.3 ± 1.4	97.3 ± 0.6
240	—[y]	—	91.5 ± 2.5	91.8 ± 1.8	94.2 ± 1.6	95.5 ± 1.7	92.4 ± 2.1	93.3 ± 2.3
360	—	—	92.8 ± 2.1	93.1 ± 1.6	92.5 ± 2.9	92.7 ± 2.0	93.6 ± 2.2	95.1 ± 1.7

[z]Data are the mean of six replicates ± SE.
[y]No capsules were obtained.

developed into healthy and normal seedlings and thus proving their fertilizing ability. This methodology has also been successful used in the cryopreservation of pollinia from two *Dendrobium* hybrids, assessing their germinability and fertilizing ability (Vendrame et al., 2008). However, in both cases, there was no significant difference either when pollinia were submitted to a PVS2 vitrification protocol or when they were properly desiccated through laminar airflow or by the silica gel method before direct storage in LN. The easy handling, simplicity of technique, and obviating the use of potentially toxic cryoprotectants make the dehydration method a preferred choice over vitrification through PVS2 exposure (Ajeeshkumar and Decruse, 2013).

In the present study, the ultralow storage of fresh pollinia was feasible without any desiccation, cryoprotection, or precooling treatment before placing directly into an ultra freezer (–70 °C) or immersing in LN (–196 °C). This is probably due to the low initial MC shown in the fresh pollinia for this species. It is interesting to consider here that orchids in general possess pollen tetrads collected into highly organized waxy pollinia with appendages. The pollen are tightly packed in the pollen sac and embedded in a highly viscous fluid, i.e., elastoviscin (Pacini and Hesse, 2002). The pollen cytoplasm and elastoviscin in pollinia are assumed to contain sucrose or other chemicals enough to protect the pollen from freezing injury. Sucrose allows pollen to be stored at low temperatures by protecting membrane integrity and through intracellular glass formation, thus preventing the formation of ice crystals (Firon et al., 2012; Speranza et al., 1997). It is assumed that the formation of highly viscous intracellular glasses decreases molecular mobility and impedes diffusion within the cytoplasm, thus slowing the deleterious reactions (Firon et al., 2012). Increased viscosity contributed by solutes concentrated in cells inhibits the coming together of water molecules to form ice, and is described as the mechanism of glass transition (Benson, 2008).

Success of a preservation protocol depends on the viability of stored material after prolonged period of storage. In this study, it has been demonstrated that pollinia of *C. cepula* stored for 360 d at –70 and –196 °C

retained pollen fertilizing ability equal to its initial level. The stored pollinia allowed successful crosses generating fruits and viable seeds which germinated and developed into healthy and normal seedlings. To our knowledge, there is no information on the in vitro germination of fresh or stored pollen from the genus *Cohniella* and this is the first report demonstrating a clear relationship between *C. cepula* pollen fertilizing ability and storage time and temperature. Additional work in our laboratory (unpublished) proved the possibility of extending this ultracold storage procedure to other wild relative species such as *Cohniella jonesiana* and *Gomesa bifolia*. Such information allows better planning of controlled breeding programs and the potential production of more diverse crosses.

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